96-well plates. Once on the plates, the cells were allowed to adhere for 3 hrs before treatment with SFU or one of the derivatives.

Cell Growth and MTT Determination of Cell Viability

HL-60 cells were treated and placed in the incubator for 24 hrs. The plates were centrifuged and the supernatant was carefully aspirated without disturbing the cell pellet. A 200 μL aliquot of media was added immediately. The cells were allowed to grow for 48 hrs. HCT-116 cells were treated and allowed to grow undisturbed for 72 hrs. The culture medium was removed by aspiration (after centrifugation in the case of HL-60 cells). 100 mL of McCoy's media and 11 μL MTT dye were added. The cells were incubated at 37°C for 3 hrs. During this time, viable cells reduce the MTT dye to purple formazan by the action of alcohol dehydrogenase. The cells were lysed by the addition of 100 μL of a solution 1.2M Hcl in 60% ethanol, thereby releasing the reduced dye into solution. The absorbance at 405 nm was measured for each well using a BIO-RAD Microplate Reader (Model 450)™/

EXAMPLE 9

In Vitro Activity of Chlorambucil-Cobalamin Bioconjugates

Thermal Stability of Bioconjugates in Media

It was noted that the chlorambucil bioconjugates **3** and **4** (prepared in Example 6) have thermal lability. Thus, they are expected to thermally decompose during the assay, perhaps before entering the cells or before release by photolysis. Thermal decomposition of both bioconjugates was monitored by a UV-vis diode array spectrophotometer (HP8452) at 37°C in water, cell-free media, and filtered media in which HCT-116 cells had been grown to a concentration of about 100,000 cells/mL. Spectra were taken hourly for a total of 8 hrs. The presence of intact bioconjugate was then determined by photolysis, 20 min, with a high-pressure mercury lamp. If photolysis had no effect on the spectrum, all of the bioconjugate was assumed to have decomposed.

In Vitro Assays of **3** and **4** Activity

Both bioconjugates were assayed against HCT-116, HL-60, B-16, Meth-A, and RD-995 cell lines. The assays were performed in the same manner as described in Example 8 as

modified herein. The B-16, RD-995 and Meth-A cells lines are all Balb/c derived carcinoma lines which were provided by Dr. R. Daynes of the University of Utah. These cell lines were grown in RPMI media which was completed with 5% fetal bovine serum and other media components as previously described. Both the B-16 and RD-995 cell lines were suspended in trypsin, as in the case of HCT-116 cells. The Meth-A cells loosely adhere to the walls of the flask and grow both attached to the flask and suspended in solution. These cells could be completely suspended by successive washing of the flask wall with media.

Assays were performed at cell concentrations of about 40,000 cells per well, with the exception of the HL-60 assay which was performed at 25,000 cell per well. The HCT-116, B-16 and RD-995 cells were assayed in flat bottomed, 96-well plates, while the HL-60 and Meth-A cells were assayed in round bottomed plates. Chlorambucil, unconjugated, was tested prior to the bioconjugates. The cells were treated with the bioconjugates in both non-photolytic and photolytic conditions. The cells were incubated for three days (media was aspirated and replaced after 24 h. in the case of the HL-60 cells) and the resulting viability measured by an MTT assay.

The MTT assay was somewhat altered for this experiment. The culture medium was aspirated after 72 hrs. The Meth-A and HL-60 cells were centrifuged prior to aspiration. Then, 100 μL of McCoy's media and 11 μL of the MTT solution were added as before. At the end of 4 hours, the culture medium was aspirated a second time (following centrifugation in the case of HL-60 and Meth-A cells) and 100 μL of DMSO was added. The DMSO lysed the cells releasing the MTT dye into solution. The absorbance of each well at 450 nm was measured as before. The HCl/ethanol solution previously used has a tendency to precipitate proteins from the resulting solution, which may give falsely increased absorbance measurements. The replacement of DMSO avoids this problem.

The concentration of chlorambucil and the bioconjugates were varied from 0.04 μM to 400 μM within the assay. The cells were treated with the bioconjugates under dim, red lights to avoid photolysis. Non-photolytic conditions were maintained by wrapping the 96 well plates with foil during the incubation periods. Photolysis was performed in black plates with flat, clear bottomed wells (Costar catalog number: 3603). These plates are sterile, collagen treated, and made of optically clear plastic. Growth of the cells in these plates did not show any differences to those grown in the normal clear plastic plates. Photolysis was achieved by an array of high

intensity green LEDs (Hewlett Packard catalog number: 782-6124). The array was constructed from one of the black plates in which one LED was placed in each well. The LEDs could be turned on and off as vertical rows. In each assay, two rows of cells were left untreated as growth controls. One of these rows was not photolyzed by the LEDs to demonstrate any unexpected effects of photolysis; irradiation did not demonstrate any effects on the untreated control cells. An empty plate was placed between the array and the assay plate to avoid heating the cells. Ten minutes of irradiation produced complete photolysis for the bioconjugate in cell-free media. The cells were irradiated for 10 min during the assay. The time of irradiation, following treatment with drug, was determined by a timecourse assay. The entire plate was treated with one of the bioconjugates at a concentration equal to the IC_{50} of chlorambucil in that cell line. The rows were irradiated separately one half hour after treatment and then hourly.

Irradiation at 1 h after treatment demonstrated the greatest bioconjugate activity in all of the cell lines. Further assays were performed with irradiation one hour after treatment. In the case of the Meth-A cell line, the cells were transferred from the round-bottomed plate into the black plate for photolysis and then returned to the round bottomed plate. The HL-60 cell line could not be tested under these photolytic conditions.

Results and Discussion

Both bioconjugates show significant thermal decomposition in both water and cell free media at 37°C. At the end of 8 hrs, photolysis has no effect on the spectrum, indicating that all of the bioconjugate has decomposed. In the HCT-116 cell media both bioconjugates show fast initial decomposition and are significantly stabilized at subsequent time. Haptocorrin, a cobalamin binding protein is known to stabilize alkylcobalamins by several orders of magnitude. This protein is present in the cell-free media from the added bovine serum. However, most of this protein in serum is saturated with cobalamin, so binding to the bioconjugates may be inhibited. It is known that several types of tumor cells secrete high amounts of cobalamin binding proteins, especially haptocorrin. Thus media in which cells have been growing has a higher concentration of apo-haptocorrin. The initial fast decomposition of the bioconjugates represents the amount remaining after the saturation of haptocorrin in the media. The bound bioconjugates are significantly stabilized by haptocorrin and the haptocorrin complex associates and dissociates in a dynamic fashion in solution, especially in the presence of significant